Plant Cell, Tissue and Organ Culture **65:** 163–167, 2001.
© 2001 *Kluwer Academic Publishers. Printed in the Netherlands.*

Research note

In vitro plant regeneration of *Mammillaria elongata* normal and cristate forms

Maria Papafotiou*, George N. Balotis, Panayiota T. Louka & John Chronopoulos
*Laboratory of Floriculture and Landscape Architecture, Department of Crop Science, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece (*requests for offprints; Fax.: +1-5294552; E-mail: mpapaf@aua.gr)*

Received 22 December 1999; accepted in revised form 30 January 2001

Key words: Cactaceae, crest, fasciation, micropropagation, tissue culture, explant seasonal response

Abstract

In the normal form of *Mammillaria elongata*, shoots were regenerated *in vitro*, through callus, from tubercle explants excised from the upper part of the branch and cultured on Murashige and Skoog medium (MS) with 1.07 μM α-napthaleneacetic acid (NAA) and 22.20 μM 6-benzylaminopurine (BA). A high percentage of tubercles explants of the *M. elongata* cristate form, excised from the tip of the branch and cultured on MS with 0.54 μM NAA and 0.44 μM BA or 1.07 μM NAA, responded by initially forming an inflated cristate shoot, which gave cristate and normal shoots, without callus intervention, when transferred on basal MS. Callus formed on cristate tubercles explants gave both cristate and normal shoots when transferred onto basal MS. Normal and cristate shoots were rooted *in vitro* on MS with 9.84 μM or 0.98 μM indole-3-butyric acid, respectively, and established *ex vitro*. In both normal and cristate form, the differential response appeared to be associated with the site of the explant excision. The formation of shoots was influenced by the season of culture; i.e., explants excised in October had a higher shoot formation rate than those excised February.

Abbreviations: BA – 6-benzylaminopurine; bT – below tip; IBA – indole-3-butyric acid; MS – Murashige and Skoog (1962) basal medium with 20 g l^{-1} sucrose; NAA – α-napthaleneacetic acid; T – tip

There are several reports on *in vitro* organogenesis of various cacti, reviewed by Fay and Gratton (1992) and Hubstenberger et al. (1992), but there are not any on tissue culture of cristate forms, although it has been pointed out that their *in vitro* propagation could be useful (Johnson and Emino, 1979a). Therefore, we were interested in the question whether a cristate form can be regenerated and maintain its form under *in vitro* conditions. Normal and cristate forms of *M. elongata*, one of the most popular cactus, have been used in the present study in order to compare their behavior *in vitro* with a view toward evaluating the role of auxins and cytokinins in their *in vitro* culture. An efficient protocol for *in vitro* propagation of *M. elongata* would minimize the need for a large number of stock plants (normal form is propagated by seed or cuttings and cristate one by cuttings or grafting), and might facilitate genetic investigation and breeding systems. In normal *M. elongata*, it has been reported that regeneration of shoots is possible via a callus stage using tubercle explants and high concentrations of cytokinins; i.e. kinetin at 93 μM, 6-(γ, γ-dimethylallylamino)purine from 49 to 295 μM and BA at 355 μM, and *in vitro* rooting at 295 μM IBA (Johnson and Emino, 1979b). In *M. elongata* var. *tenuis*, shoot regeneration was achieved only when stem slices of flowering shoots were used as explants (Mehra and Cheema, 1980). A number of other species of *Mammillaria* have been regenerated *in vitro* (Fay and Gratton, 1992; Hubstenberger et al., 1992) and each of these species required a specific and unique auxin-cytokinin combination for specific responses.

In the present work, explants of the normal form were single tubercles excised (with part of the branch

tissue at their base) either from the apical part of a 5-cm-long branch (2cm zone from the tip, excluding the tip), or from the middle (2–3.5 cm below the tip), or the basal part (3.5–5 cm below the tip) of the branch. Explants of the cristate form consisted of branch slices bearing tubercles excised from the tip (T) or from the 1 cm zone below the tip (bT) of the branch; T explants bear four tubercles and bT explants three. Tubercle explants of both normal and cristate forms were excised from two-year-old stock plants grown from shoot cuttings in an unheated greenhouse. Branches dissected from the stock plants had their spines trimmed and then were immersed in 95% ethanol for 30 sec followed by immersion in sterile distilled water and then surface sterilized in 10% of commercial bleach (4.5% sodium hypochlorite) with 0.1% polyoxyethylenesorbitan for 15 min and followed by a further 3-min rinse, three times with sterile distilled water. Explants were placed in culture in late October and late February; the average monthly temperature for these months in the greenhouse where the stock plants were kept was 22 °C and 10 °C, respectively.

The explants were cultured in 80 ml jars (15 ml medium, three explants per jar) on solid MS (8 g l^{-1} agar) and various concentrations of NAA and BA mentioned in the relevant sections below (pH= 5.6 before autoclaving). The explants were incubated at 25 °C and a 16-h photoperiod of 37.5 μmol m^{-2} s^{-1} fluorescent light. In all experiments, data was collected after two months of culture, except assessment for rooting that was made after 20 days. Fifteen or eighteen explants were used per treatment, in a completely random design. The significance of the results was tested by two-way analysis of variance (ANOVA) and the means of the treatments were compared by the Tukey-Kramer HSD at $p = 0.05$ (JMP software, SAS Institute Inc.). All experiments were repeated twice with similar results; the data of one experiment is presented.

Tubercle explants from the apical part of the branch of the normal *M. elongata* were cultured on MS media containing all possible combinations of 1.07, 5.37 or 26.85 μM NAA with 11.09, 22.19 or 44.39 μM BA. All the above combinations induced callus proliferation initially and subsequently, after one month of culture on the initial medium, shoot regeneration on the callus tissue. A higher percentage of tubercles gave shoots when excised in October than in February, while more shoots per explant were formed in 1.07 μM NAA and 11.09 or 22.19 μM BA in October or 44.39 μM BA in February (Table 1). Tubercles excised from the apical part of the branch gave a higher number of shoots per explant (0.9±0.15) than those from the middle (0.5±0.18), while tubercles from the basal part produced only callus, when cultured on media with 1.07 μM NAA and 11.09 or 22.19 μM BA (mean numbers are average for both media and seasons).

Table 1. Percentage of normal *M. elongata* tubercle explants respondind by forming shoots and mean number of shoots on all explants, when cultured on MS with the NAA and BA concentrations shown, in October and February, after 60 days of culture

Concentration (μM)		Shoots (%)		Shoot no.	
NAA	BA	Oct.	Feb.	Oct.	Feb.
1.07	11.09	50ab	50ab	1.3b	0.7ab
5.37	11.09	50ab	22a	0.5ab	0.2a
26.85	11.09	0a	0a	0.0a	0.0a
1.07	22.19	88b	35ab	1.7b	0.9ab
5.37	22.19	35ab	0a	0.5ab	0.0a
26.85	22.19	0a	64b	0.0a	0.6ab
1.07	44.39	35ab	35ab	0.4a	1.2b
5.37	44.39	64ab	11a	0.6ab	0.1a
26.85	44.39	0a	11a	0.0a	0.1a

Mean separation in column by Tukey-Kramer HSD at p=0.05.

Media that gave a good response of shoot formation in normal *M. elongata* tubercle explants (MS with 1.07 μM NAA and 11.09 or 22.19 μM BA) induced only light green, hyperhydrated callus in tubercles explants of the cristate form. Therefore, lower concentrations of NAA and BA were tested; i.e., all possible combinations of 0, 0.54 or 1.07 μM NAA with 0, 0.44 or 0.89 μM BA. Almost 100% of T explants and 50–70% of bT explants responded forming either one inflated cristate shoot, or one normal shoot, or callus only. Inflated cristate shoots or normal shoots developed directly on the explants, without callus intervention. In some explants, the inflated cristate shoots co-existed with callus. After two months of culture, the inflated cristate shoots were transferred onto basal MS and cristate and normal shoots then developed on them (Figure 1). The fact that much lower BA concentrations were needed for shoot formation in the cristate form than the normal one is aligned with the finding that fasciated tissues had an increased level of free cytokinins (Nilsson et al., 1996). In October, almost all T explants gave one inflated cristate shoots or callus, while many explants from bT gave one normal shoot (Table 2a). This response resembles

Table 2. (a) Percentage of tubercles explants of *M. elongata* cristate form, from the tip (T) or 1 cm below the tip (bT) of the branch, responded forming one inflated cristate shoot (i.cr.sh.), or one normal shoot, when cultured in October on MS with the NAA and BA concentrations shown, 60 days after the beginning of culture; and mean number of cristate and normal shoots formed on all these i.cr.shs., when transferred on basal MS, 60 days after the transfer; (b) Percentage of tubercles explants of *M. elongata* cristate form, from the tip of the branch, responded forming one i.cr.sh., or one normal shoot, or callus only, when cultured in February on MS with the NAA and BA concentrations shown, 60 days after the beginning of culture; and mean number of cristate and normal shoots formed on all these i.cr.shs and callus, when transferred on basal MS, 60 days after the transfer

(a)

Concentration (μM)		Inflated cristate shoot (%)		Normal shoot (%)		Cristate shoot number		Normal shoot number	
NAA	BA	T	bT	T	bT	T	bT	T	bT
0.00	0.00	38ab	17ab	13a	33ab	2.0bc	1.5b	9.0abc	3.5ab
0.54	0.00	33a	6a	0a	27ab	1.0abc	2.0b	8.0abc	4.5ab
1.07	0.00	100b	17ab	0a	17ab	2.0c	0.0a	4.0a	0.0a
0.00	0.44	50ab	22ab	33b	33ab	0.9ab	0.5a	4.5abc	7.5ab
0.54	0.44	100b	25ab	0a	53b	1.5bc	0.0a	10.3c	0.0a
1.07	0.44	33a	11ab	0a	17ab	0.0a	0.5a	0.0a	4.0ab
0.00	0.89	100b	–	0a	–	2.0bc	–	5.3abc	–
0.54	0.89	75ab	27ab	17ab	20ab	1.0abc	0.0a	7.2abc	8.0ab
1.07	0.89	50ab	8ab	0a	17ab	0.3a	1.0b	0.0a	7.0ab

(b)

Concentration (μM)		Inflated cristate shoot (%)	Normal shoot (%)	callus	Cristate shoot number		Normal shoot number	
NAA	BA	T	T	T	Inflated cristate shoot	Callus	Inflated cristate shoot	Callus
0.00	0.00	15ab	54b	15a	1.0a	2.0a	3.5a	2.0a
0.54	0.00	13a	53b	27ab	0.5a	1.8a	1.0a	3.5a
1.07	0.00	60ab	20a	20a	1.3a	1.0a	3.7a	6.7a
0.00	0.44	71b	0a	21ab	1.1a	0.8a	4.9ab	7.5a
0.54	0.44	67b	8a	25ab	1.8a	1.7a	3.6ab	6.7a
1.07	0.44	50ab	0a	50ab	1.4a	1.6a	7.6ab	6.0a
0.00	0.89	47ab	0a	53ab	1.6a	1.8a	5.7b	6.4a
0.54	0.89	29ab	7a	64b	1.3a	1.9a	8.7b	6.2a
1.07	0.89	29ab	0a	71b	2.0a	1.0a	7.2b	6.3a

Mean separation in column by Tukey-Kramer HSD at p=0.05.

the *in vivo* behaviour of other cristate cacti. In *Echinocereus reichenbachii*, if the cristate meristem was removed, normal shoots were produced from the lateral tubercles; when smaller portions were excised, there was renewed activity in the cristate meristem (Boke and Ross, 1978). In February, the bT explants had a similar response to October explants, so data is not shown, while the percentage of T explants that gave inflated cristate shoots was reduced. In media without BA, a lot of T explants gave one normal shoot. Addition of 0.44 μM BA promoted inflated cristate shoots formation where 0.89 μM BA promoted callus formation (Table 2b). Cytokinins applied exogenously have been found to cause fasciation to *Pisum sativum* (Thimann and Sachs, 1966) and *Kalanchoe blossfeldiana* (Varga et al., 1988). Media supplemented with 1.07 μM NAA or with 0.54 μM NAA and 0.44 μM BA, in both seasons, induced a high percentage of inflated cristate shoots formation in T explants and a high number of cristate shoot regeneration on the inflated cristate shoots when these inflated cristate shoots were transferred onto basal MS (Table 2a, b). The medium supplemented with 0.89 μM BA also promoted cristate formation, but induced hyperhydration (50%) which was induced in all media that contained 0.89 μM BA.

Fifty or 67% of shorter than 1 cm normal or cristate shoots, respectively, rooted *in vitro* on MS with 9.84 or 0.98 μM IBA, respectively, and acclimatized with 90–100% success *ex vitro* in a peat:perlite medium (1:2

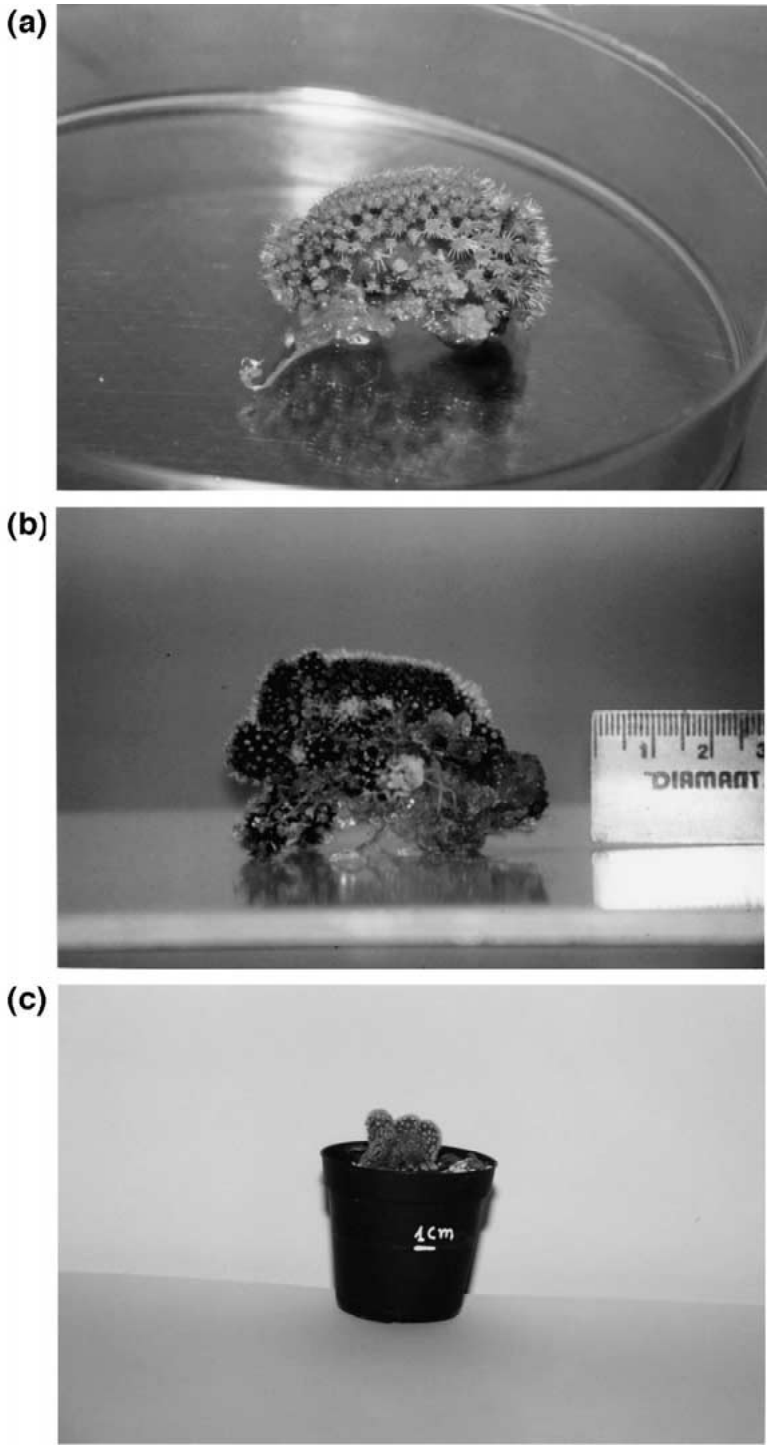

Figure 1. (*a*) An inflated cristate shoot initiated on tubercles explant of *M. elongata* cristate form, after two months of culture on MS with 0.54 μM NAA and 0.44 μM BA. (*b*) Cristate and normal shoots developed on an inflated cristate shoot, after 4 months in culture (2 months in growth regulator containing medium and 2 months in basal MS). (*c*) Cristate *M. elongata* plant regenerated *in vitro* and growing in peat:perlite (1:2 by volume), after one year.

by volume). Shoots longer than 1 cm rarely rooted *in vitro,* but rooted *ex vitro* in the above medium with a 50–60% success rate. The *in vitro* regenerated plants were all phenotypically identical to the stock plants (Figure 1). Two years after recovery, 80% of the cristate plants retained the cristate appearance while the rest developed some normal shoots.

Some of the explants of the cristate form produced only callus. Callus formed on different media responded in a similar way, when transferred onto basal MS, by forming cristate and normal shoots that were almost all hyperhydrated. The season or the explant site did not affect the response; thus, only the February data for T explants is presented (Table 2b). The different concentrations of plant growth regulators in the media, where the callus was formed, did not seem to affect the form of shoots regenerated on the callus; however, the number of callus was small and differed in each treatment. Therefore, shoot formation in the cristate *M. elongata* was observed both directly on the explants and through callus. It has been suggested that in cristate forms "a genetic mechanism might operate through a hormonal imbalance that is restricted to the linear meristem and its immediate vicinity" (Boke and Ross, 1978). The results of the present work partly support the above hypothesis; however, the simultaneous regeneration of cristate and normal shoots from callus is difficult to be interpreted. Further work *in vitro* with cristate forms could help to the understanding of the phenomenon of fasciation.

References

Boke NH & Ross RG (1978) Fasciation and dichotomous branching in *Echinocereus* (Cactaceae). Amer. J. Bot. 65(5): 522–530

Fay MF & Gratton J (1992) Tissue culture of cacti and other succulents: a literature review and a report on micropropagation at Kew. Bradleya 10: 33–48

Hubstenberger JF, Clayton PW & Phillips GC (1992) Micropropagation of Cacti. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol 20 (pp. 49–68). Springer-Verlag, Berlin

Johnson JL & Emino ER (1979a) Tissue culture propagation of cacti. Cact. Succ. J. (US) 51: 275–277

Johnson JL & Emino ER (1979b) *In vitro* propagation of *Mammillaria elongata*. HortScience 14(5): 605–606

Mehra PN & Cheema GS (1980) *In vitro* studies on *Mammillaria elongata* var. *tenuis*. Phytomorphology 30: 241–249

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nilsson O, Moritz T, Sundberg B, Sandberg G & Olsson O (1996) Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fasciation. Plant Physiol. 112: 493–502

Thimann KV & Sachs T (1996) The role of cytokinins in the 'fasciation' disease caused by *Corynebacterium fascians*. Am. J. Bot. 53: 731–739

Varga A, Thoma H & Bruinsma J (1988) Effects of auxins and cytokinins on epigenetic instability of callus-propagated *Kalanchoe blossfeldiana* Poelln. Plant Cell Tiss. Org. Cult. 15: 223–231